Nephrol Dial Transplant (2009) 24: 2755–2762
doi: 10.1093/ndt/gfp152
Advance Access publication 8 April 2009

Patients with IgA nephropathy exhibit high systemic PDGF-DD levels

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Abstract

Background. Platelet-derived growth factor (PDGF) is a central mediator of mesangioproliferative glomerulonephritis (GN). In experimental mesangioproliferative GN, PDGF-DD serum levels, unlike PDGF-BB, increased up to 1000-fold.

Methods. We assessed disease activity in 72 patients with GN, established a novel PDGF-D ELISA and then determined their PDGF-DD levels. In parallel, we studied renal PDGF-DD mRNA expression by RT-PCR.

Results. PDGF-DD serum levels in patients with IgA nephropathy (IgAN) were significantly higher (1.67 ± 0.45 ng/ml) and in patients with lupus nephritis significantly lower (0.66 ± 0.86 ng/ml) compared to healthy controls (1.17 ± 0.46 ng/ml), while patients with focal segmental glomerulosclerosis, membranous GN and ANCA-positive vasculitis did not differ from controls. The subgroup of IgAN patients with elevated PDGF-DD levels (27% of samples) did not differ in their clinical features from those with normal PDGF-DD levels. In IgAN patients with repetitive PDGF-DD determinations, most exhibited only minor fluctuations of serum levels over time. Intrarenal PDGF-DD mRNA expression did not differ between controls and patients, suggesting an extrarenal source of the elevated PDGF-DD in IgAN.

Conclusions. Serum PDGF-DD levels were specifically elevated in patients with IgAN, in particular in those with early disease, i.e. preserved renal function. Our data support the rationale for anti-PDGF-DD therapy in mesangioproliferative GN.

Keywords: glomerulonephritis; growth factor; serum marker

Introduction

Ample evidence links members of the platelet-derived growth factor (PDGF) family to the pathogenesis of renal diseases, in particular mesangioproliferative glomerulonephritis (GN) (reviewed in [1]). PDGF-DD, like PDGF-BB, is a major mitogen for mesangial cells that largely signals through the PDGF receptor (PDGFR)-β [1] and is crucially involved in the development of mesangioproliferative GN. PDGF-DD, which is not normally expressed in glomeruli, was prominently overexpressed in the mesangial region following the induction of mesangioproliferative anti-Thy 1.1 nephritis in rats [2]. In mice, hepatic viral overexpression of a human PDGF-DD construct induced mesangioproliferative changes [3]. Finally, in mesangioproliferative anti-Thy 1.1 nephritis glomerular cell proliferation, matrix accumulation and subsequent glomerulosclerosis and tubulointerstitial damage were all significantly reduced by a specific neutralizing anti-PDGF-DD monoclonal antibody [2,4]. Similar findings had also previously been described in the case of PDGF-BB [1]. However, whereas PDGF-BB exerts mostly autocrine and paracrine short-range actions, we noted during the course of experimental mesangioproliferative nephritis in rats that PDGF-DD plasma levels increased ∼1000-fold [2]. Thus, unlike PDGF-BB, PDGF-DD apparently also acts as an endocrine growth factor [2,5] and might represent a non-invasive marker for mesangioproliferative GN. This observation prompted us to investigate the PDGF-DD expression in patients with renal diseases and in particular the question of whether PDGF-DD serum levels in patients with glomerular disease, especially mesangioproliferative IgA nephropathy (IgAN), can serve as a biomarker of disease and/or disease activity.

Subjects and methods

Patients and serum samples

Sample collection and the clinical study were approved by the local Institutional Review Board and were performed according to the Declaration of Helsinki. All serum samples and clinical parameters were prospectively obtained during scheduled outpatient clinic visits at the University Hospital Aachen. Once drawn, blood samples

Table 1. Patient characteristics

Disease entity	IgAN[a] ($n = 33$)	LN ($n = 18$)	FSGS ($n = 10$)	VAS ($n = 8$)	MN ($n = 12$)
Serum PDGF-DD (ng/ml)	1.67 ± 0.45	0.66 ± 0.86	0.85 ± 0.36	1.12 ± 0.66	1.26 ± 0.64
	1.70 (0.56–2.72)	0.28 (0.06–3.15)	0.87 (0.41–1.33)	0.88 (0.39–2.36)	1.25(0.27–2.68)
Age (years)	40 ± 13	45 ± 16	38 ± 14	59 ± 14	52 ± 16
	39 (19–78)	44 (20–77)	38 (18–63)	60 (41–76)	46 (25–77)
Sex (male:female)	24: 9	4: 14	5: 5	7: 1	6: 6
Body weight (kg)	80 ± 18	73 ± 19	82 ± 17	87 ± 10	89 ± 27
	83 (51–120)	70 (45–110)	81 (63–107)	91 (73–99)	84 (64–170)
BMI (kg/m^2)	26 ± 5	25 ± 5	22 ± 19	28 ± 4	32 ± 11
	25 (20–38)	24 (17–35)	25 (21–40)	28 (23–34)	30 (23–65)
SBP (mmHg)	138 ± 17	123 ± 13	133 ± 20	139 ± 25	142 ± 18
	135 (109–170)	125 (100–140)	140 (90–160)	133 (115–180)	145 (115–170)
DBP (mmHg)	83 ± 11	74 ± 8	88 ± 13	80 ± 16	79 ± 14
	80 (65–120)	75 (60–90)	88 (70–110)	85 (60–105)	78 (55–100)
No. of anti-hypertensive drugs	1.6 ± 1.0	1.3 ± 1.3	1.5 ± 1.0	1.5 ± 0.8	2.3 ± 1.4
	1 (0–4)	1 (0–4)	1 (0–3)	1 (1–3)	2 (0–4)
Immunosuppression	6 (18%)	18 (100%)	3 (30%)	3 (38%)	5 (42%)
Serum creatinine (mg/dl)	2.0 ± 1.5	1.4 ± 0.9	1.5 ± 0.5	1.9 ± 0.6	1.9 ± 1.0
	1.5 (0.8–8.4)	1.1 (0.7–4.5)	1.5 (0.8–2.2)	1.8 (1.2–3.2)	1.5 (0.9–3.5)
Serum total protein (g/l)	68 ± 7	67 ± 10	59 ± 12	67 ± 5	55 ± 10
	68 (52–86)	68 (42–80)	63 (41–73)	69 (56–73)	57 (40–70)
Serum CRP (mg/l)	4 ± 8	5 ± 6	2 ± 4	24 ± 39	3 ± 4
	0 (0–43)	6 (0–16)	0 (0–10)	3 (0–88)	0 (0–11)
(G/l)	7.7 ± 2.1	8.5 ± 4.6	7.9 ± 2.2	7.8 ± 3.5	13.7 ± 20.1
	7.5 (4.2–11.9)	6.9 (4.0–21.2)	7.6 (5.2–12.0)	6.0 (4.4–13.6)	7.4 (4.4–74.0)
Creatinine clearance (ml/min)	71 ± 39	72 ± 47	57 ± 40	65 ± 35	79 ± 48
	72 (20–195)	54 (13–156)	45 (17–124)	59 (29–129)	80 (12–157)
24-h proteinuria (g/day)	2.5 ± 2.6	1.1 ± 1.4	3.2 ± 2.1	1.1 ± 0.7	5.6 ± 6.4
	2.3 (0–9.8)	0.4 (0–4.1)	3.0 (0.2–6.4)	0.9 (0.4–2.1)	2.2 (0–19.1)
U-protein to creatinine (mg/g × 10^3)	2.4 ± 2.1	5.6 ± 7.5	2.9 ± 2.1	0.8 ± 0.5	9.5 ± 6.4
	1.9 (0.2–6.7)	5.6 (0.3–10.8)	3.5 (0.2–6.5)	0.8 (0.4–1.6)	10.6 (0.9–16.3)
U-albumin to U-creatinine (mg/g × 10^3)	1.9 ± 1.6	3.9 ± 5.5	2.3 ± 1.6	0.6 ± 0.5	5.7 ± 4.2
	1.5 (0.1–5.2)	3.9 (0.1–7.7)	2.3 (0.1–4.8)	0.4 (0.2–1.4)	6.1 (0.7–10.2)
LDH (U/l)	173 ± 35	228 ± 62	213 ± 61	213 ± 95	247 ± 107
	166 (123–271)	205 (131–354)	193 (157–352)	182 (137–429)	218 (147–517)
Haematuria—none	23%	53%	70%	50%	40%
(+)	27%	27%	20%	12.5%	50%
+	15%	20%	10%	12.5%	10%
++	32%	0%	0%	25%	0%
+++	3%	0%	0%	0%	0%

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure.
[a]In the case that IgAN patients' PDGF-DD serum levels measured in the first available sample entered the table.
All values are means ± SD in the upper rows and median (minimum–maximum) in the lower rows or percent of patients.

were separated and sera were stored immediately at −80°C until the assay.

A total of 167 serum samples were obtained from patients with the following biopsy-confirmed diagnoses: primary IgAN (137 samples from 33 patients with the follow-up from 0 to 52 months), focal segmental glomerulosclerosis (FSGS; 10 samples from 10 patients), ANCA-positive vasculitis (VAS; 8 samples from 8 patients) and membranous nephropathy (MN; 12 samples from 12 patients). We also analysed 18 sera from patients with lupus nephritis (LN), of which 9 had biopsy-proven LN (1 patient with WHO class I, 2 patients with class II, 3 patients with class IV and 2 patients with class VI), whereas the remaining 9 had suspected nephritis due to lupus erythematosus and proteinuria. We excluded patients with secondary GN forms and active infections. At the time of each outpatient visit, the parameters listed in Table 1 were assessed. Proteinuria and creatinine clearance were measured based on 24-h urine collections. Immunosuppression was defined as any type of immunosuppressive medication at the time of obtaining the blood sample, i.e. corticosteroids, cyclophosphamide, cyclosporine A, tacrolimus, azathioprine or combinations thereof.

Forty-four healthy blood donors with a mean age of 38 ± 13 years (median age 38, range 21–63 years), 26 men and 16 women, served as normal controls (only age and sex were available for this group). It has been previously shown that patients with lung cancer have elevated serum PDGF-DD levels [6]. Therefore, we have analysed 18 samples from patients with various types of lung cancer as a positive control for the ELISA.

ELISA for PDGF-DD in human serum

A specific sandwich ELISA was developed to measure human serum PDGF-DD. The antibodies used for the ELISA were provided by Curagen Corp. (Branford, CT, USA). Briefly, the ELISA was performed as follows: 100 μl of capture antibody (fully human monoclonal anti-PDGF-DD Ab; 0.25 μg/ml) in a coating buffer [0.05 M $NaHCO_3$ (pH 9.6)] was coated on ELISA plates. After overnight incubation at 4°C, the plates were washed (four times) using 0.05% Tween 20 in PBS (washing buffer) and 225 μl of blocking buffer (0.5% BSA, 0.1% Tween 20, 0.01% Thimerosal in PBS) was added for 1 h at 25°C. After washing, 100 μl of standards and serum samples were added and plates were incubated at 37°C for 1 h. After washing again, 1-h incubation at 37°C with 100 μl of detection antibody followed (horseradish peroxidase-conjugated rabbit polyclonal anti-human-PDGF-DD Ab; 0.3 μg/ml). After washing, the plates were incubated for 10 min at room temperature in the dark with 100 μl of HRP substrate (TMB reagent, R&D) and the reaction was stopped with 100 μl of 1 N H_2SO_4. The plates were analysed on an ELISA plate reader at 450 nm with a correction at 570 nm. Serum PDGF-DD concentrations were calculated from a PDGF-DD standard curve using a four-parameter curve-fitting program. Detection and capture antibodies detected both

latent and active PDGF-DD, i.e. PDGF-DD with and without CUB domain, respectively (data not shown).

Western blot or PDGF-DD in human serum

PDGF-DD western blotting was performed in six healthy subjects and eight IgAN patients as previously described with minor modifications [2]. Briefly, sera were diluted in a SDS–PAGE sample buffer to obtain equal protein content, and 20 μl were then subjected to 10% SDS–PAGE under reducing conditions. Proteins were transferred to Hybond-P membranes (Amersham), and filters were probed with affinity-purified rabbit polyclonal anti-PDGF-DD Ab (kindly provided by Dr Eriksson, Ludwig Institute for Cancer Research, Stockholm, Sweden) or control rabbit IgG for 12 h. After washing, filters were incubated with an anti-rabbit HRP secondary antibody. Bands were visualized by enhanced chemiluminescence (Amersham).

Microdissection, RNA isolation, real-time PCR analyses

Human kidney biopsies from healthy living kidney donors (LD; $n = 9$), patients with IgAN ($n = 24$), LN ($n = 11$), FSGS ($n = 13$), rapidly progressive GN (RPGN; $n = 9$) or with MN ($n = 22$), were obtained in a multicentre study for gene expression analysis in renal biopsies (see the Acknowledgements for participating centres). Informed consent of the patients and agreements of the local ethics committees were obtained. For a detailed description of the protocol used see [7]. In brief, directly after biopsy a cortical tissue segment was transferred into a commercially available RNase inhibitor (RNAlater, Ambion, TX, USA), stored at −20°C followed by manual microdissection to separate glomeruli and tubulointerstitial compartments in RNAlater. Total RNA was isolated using a commercially available silica-gel-based isolation protocol and underwent reverse transcription. Real-time PCR (RT-PCR) was performed on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Darmstadt, Germany). Glomerular microdissection was confirmed by RT-PCR for the glomerulus-specific cDNA for Wilms tumour antigen 1. A predeveloped, cDNA-specific assay for PDGF-DD (Hs00937332_ml, Applied Biosystems) was used, and the PDGF-DD mRNA expression was normalized to two reference genes (GAPDH and 18S rRNA, both predeveloped assays from Applied Biosystems). Both reference genes gave comparable results. Water and no-template controls were negative in all runs.

Statistics

Data are given as means ± SD and median (minimum–maximum). Since the Kolmogorov–Smirnov test indicated a non-normal distribution of the data, we applied non-parametric tests (Kruskal–Wallis *H*-test and Mann–Whitney *U*-test) to compare patient groups and Spearman's correlation to analyse the association between the tested variables. We used the χ^2 test to compare categorical variables. All tests were two-tailed, and statistical significance was defined as $P < 0.05$.

Results

Characteristics of the PDGF-DD ELISA and circulating PDGF-DD

The lower quantification limit of the PDGF-DD ELISA was 0.2 ng/ml. Intra- and inter-day precisions were assessed by serial measurements, and in repetitive runs, yielded coefficients of variation of 1.0–8.7% and 6.3–8.7%, respectively. Recovery of PDGF-DD from naïve serum was 94.1, 90.2 and 99.5% at 0.6, 5.0 and 40.0 ng/ml PDGF-DD, respectively. Serum concentrations were stable within three freeze-thaw cycles.

In healthy blood donors, PDGF-DD levels were 1.17 ± 0.46 ng/ml. The normal range was set as mean ± 2 SD: 0.25–2.09 ng/ml (Figure 1). The 5th and 95th percentiles were similar at 0.43 and 2.01 ng/ml, respectively.

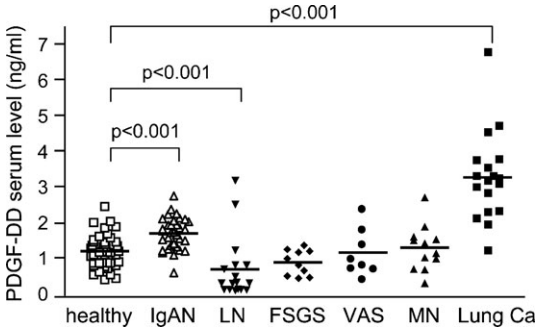

Fig. 1. Individual serum PDGF-DD concentrations in healthy subjects, in patients with glomerular diseases and in patients with lung cancer. The horizontal lines depict the mean values of the groups. The following groups are shown: healthy controls (healthy), patients with IgA nephropathy (IgAN), lupus nephritis (LN), focal segmental glomerulosclerosis (FSGS), ANCA-positive vasculitis (VAS), membranous nephropathy (MN) and various types of lung cancer (Lung Ca). The Kruskal–Wallis *H*-test indicated significant differences ($P < 0.001$) between the groups. Differences between groups were evaluated using the Mann–Whitney *U*-test.

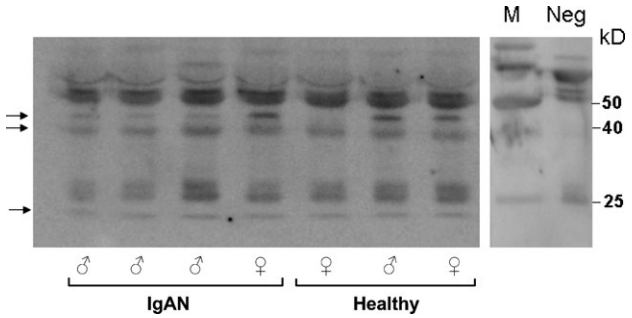

Fig. 2. Representative PDGF-DD western Blot analysis under reducing conditions with sera from four IgAN patients and three healthy controls. The black arrows label specific bands that most likely represent the monomer plus CUB domain (about 47 kD), the dimer form without CUB domain (about 34 kD) and the monomer without CUB domain (about 22 kD). IgAN patients and healthy controls exhibited no obvious differences in molecular characteristics of their circulating PDGF-DD. M = size marker, Neg = negative control (blot was incubated with irrelevant rabbit IgG instead of the specific rabbit anti-PDGF-D-Ab).

PDGF-DD levels were markedly elevated in patients with lung cancer that were included as 'positive' controls (Figure 1) [6].

To assess whether the molecular characteristics of circulating PDGF-DD are altered in patients with renal disease, we performed western blotting under reducing conditions (Figure 2). We detected three specific bands of ∼47, 35 and 22 kD, most likely representing monomeric PDGF-DD including its CUB domain (47 kD), the dimer form without a CUB domain (34 kD) and the monomer without a CUB domain (22 kD) [2,5]. Sera from patients and controls did not differ. Furthermore, no differences were detected between IgAN patients with different serum creatinine and PDGF-DD concentrations.

PDGF-DD levels in different glomerular diseases

The clinical characteristics of the patients are given in Table 1. When compared with healthy subjects, serum PDGF-DD concentrations were similar in patients with FSGS, MN and VAS. Patients with IgAN and LN exhibited

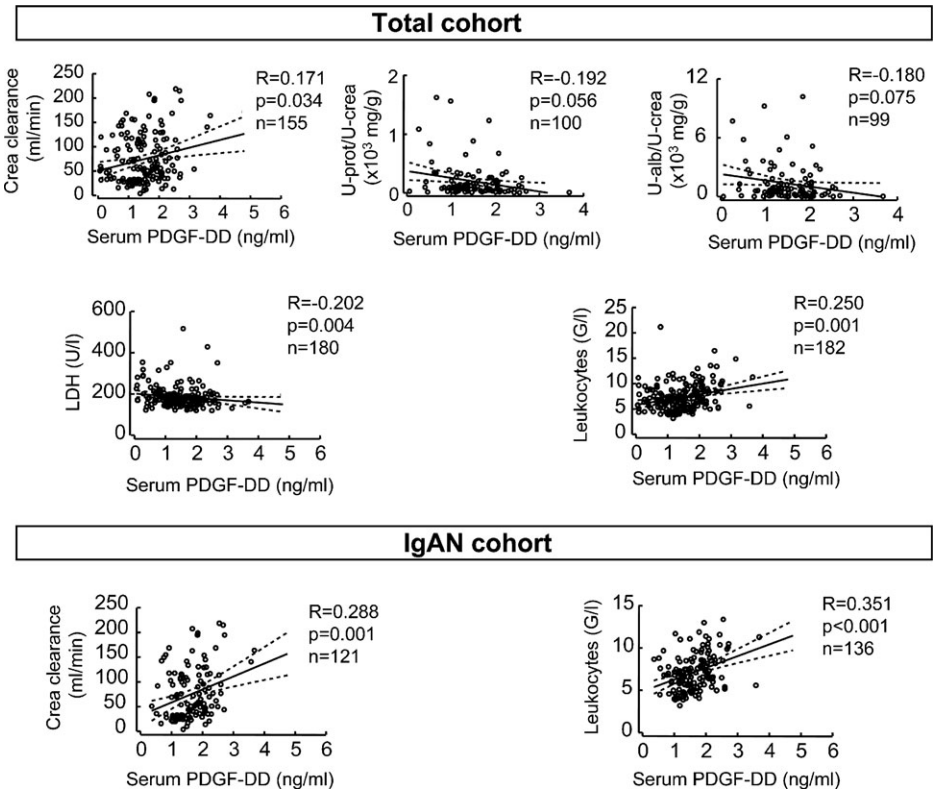

Fig. 3. Correlation between PDGF-DD serum levels and creatinine clearance, urinary protein or albumin to creatinine ratios, LDH and leukocytes in the whole cohort and in patients with IgAN. Each graph shows the individual values, linear regression line (full line) and the 95% confidence interval band of the regression line (dashed lines), Spearman correlation coefficient (R), statistical significance (p) and the number of subjects analysed (n). Crea clearance = creatinine clearance; U-prot./U-crea. = urinary protein to creatinine ratio; U-alb./U-crea = urinary albumin to creatinine ratio.

significantly higher and lower concentrations, respectively, than normal controls (Table 1 and Figure 1).

PDGF-DD serum levels were comparable in males and females of the whole group and the subgroups of healthy subjects and patients with LN, FSGS and MN (data not shown). In contrast, male IgAN patients exhibited higher serum PDGF-DD than females (1.76 ± 0.48 ng/ml, median 1.86, range 0.56–2.72 in 24 male patients versus 1.44 ± 0.25, 1.38 and 1.12–1.74 in 9 female patients, respectively; $P = 0.036$).

As can be seen in Figure 1, all LN patients exhibited low PDGF-DD serum levels apart from two patients, both of whom had advanced sclerosing LN (class VI).

We detected no correlation between age and serum PDGF-DD concentrations in the whole population or in the disease subgroups.

Effects of proteinuria and renal function on PDGF-DD serum levels

In the whole cohort of renal patients (including the follow-up samples), serum PDGF-DD concentrations correlated positively with creatinine clearance ($P = 0.034$) and leukocyte counts ($P = 0.001$) and negatively with lactate dehydrogenase (LDH; $P = 0.004$), a non-specific marker of cell damage (Figure 3). Urinary protein to urinary creatinine ratios and urinary albumin to urinary creatinine ratios showed a trend to be negatively correlated with serum PDGF-DD levels, but the correlation failed to reach statistical significance (Figure 3). No other measured variables of renal function correlated with PDGF-DD serum levels.

To extend these observations, we divided the cohort into four groups according to 25th percentiles of PDGF-DD concentrations (Table 2). In this analysis, creatinine clearance was highest in the patient percentile with the highest PDGF-DD serum concentrations. Notably, the patients in the lowest PDGF-DD percentile also had a relatively high clearance creatinine. A negative correlation of PDGF-DD serum levels with LDH concentrations was confirmed in this analysis. Leukocyte counts were significantly elevated in the PDGF-DD group with the lowest values. Finally, by χ^2 testing the number of patients receiving immunosuppression was significantly higher in the 0–25th percentile group (Table 2).

IgAN

In patients with IgAN (including the follow-up samples), we found PDGF-DD to be significantly and positively correlated with creatinine clearance and leukocyte counts (Figure 3). These findings were uniformly confirmed after categorization of IgAN patients into groups according to PDGF-DD percentiles (Table 3).

When we separated the IgAN patients based on PDGF-DD concentrations below or above the upper normal limit (see above), we confirmed the association

Table 2. Division of the whole patient cohort into four groups based on 25th percentiles of serum PDGF-DD concentrations

Percentile	0–25th ($n = 44$)	25th–50th ($n = 38$)	50th–75th ($n = 51$)	75th–100th ($n = 52$)	P-value
Serum PDGF-DD (ng/ml)	<0.96	0.97–1.32	1.33–1.85	>1.86	
Creatinine clearance (ml/min)	$79 \pm 48^{\S\S}$	55 ± 38	72 ± 53	$87 \pm 54^{\#}$	0.016
	65 (16–169)	33 (20–156)	54 (4–208)	73 (13–219)	
(G/l)	$8.8 \pm 10.6^{\S,\$,\&}$	6.3 ± 2.2	6.8 ± 2.1	8.8 ± 2.7	<0.001
	6.8 (4.0–74.0)	6.3 (3.2–12.0)	6.4 (4.1–11.9)	8.5 (5.0–16.5)	
LDH (U/l)	$204 \pm 53^{\S,\$,\&}$	177 ± 46	183 ± 59	179 ± 53	0.005
	197 (123–354)	168 (123–352)	171 (123–517)	168 (120–429)	
Immunosuppression*	8.4%	2.2%	3.1%	6.2%	0.006

P-values of Kruskal–Wallis H-tests for the numeric parameters or the χ^2 test for immunosuppression are shown. The data are given as means ± SD in the upper row and medians (minimum–maximum) in the lower row or as percentage of patients receiving immunosuppression.
*All patients, except for three receiving cyclosporine A as monotherapy, were treated with corticosteroids as mono- or combination therapy.
The Mann–Whitney U-test was used to detect differences between individual groups.
0–25th versus 25th–50th: $^{\S}P < 0.01$ or $^{\S\S}P < 0.05$; 0–25th versus 50th–75th: $^{\$}P < 0.01$ or $^{\$\$}P < 0.05$; 0–25th versus 75th–100th: $^{\&}P < 0.01$; 75th–100th versus 25th–50th: $^{\#}P < 0.01$; 75th–100th versus 50th–75th: $^{*}P < 0.05$.

Table 3. Division of IgA nephropathy patients into four groups according to 25th percentiles of serum PDGF-DD concentrations

Percentile	0–25th ($n = 13$)	25th–50th ($n = 34$)	50th–75th ($n = 42$)	75th–100th ($n = 48$)	P-value
Serum PDGF-DD (ng/ml)	< 0.96	0.97–1.32	1.33–1.85	>1.86	
Creatinine clearance (ml/min)	91 ± 56	51 ± 33	72 ± 55	$91 \pm 53^{\#,**}$	0.003
	98 (16–169)	32 (20–118)	51 (4–208)	77 (14–219)	
(G/l)	6.9 ± 1.6	6.1 ± 2.0	6.7 ± 2.0	$8.4 \pm 2.2^{\&,\#,*}$	<0.001
	6.8 (4.5–9.7)	6.0 (3.2–11.7)	6.2 (4.1–11.9)	8.4 (5.0–13.4)	

Kruskal–Wallis H-test P-values are shown. The data are given as means ± SD in the upper rows and medians (minimum–maximum) in the lower rows.
The Mann–Whitney U-test was used to detect differences between individual groups.
0–25th versus 25th–50th: $^{\S}P < 0.05$; 75th–100th versus 0–25th: $^{\&}P < 0.05$; 75th–100th versus 25th–50th: $^{\#}P < 0.01$; 75th–100th versus 50th–75th: $^{*}P < 0.01$ or $^{**}P < 0.05$.

Table 4. IgA nephropathy patients; comparison of serum samples with PDGF-DD concentrations above or below the upper limit of normal

Serum PDGF-DD (ng/ml)	<2.01	>2.01	P-value
n	100	37	
DBP (mmHg)	82 ± 9	75 ± 8	0.001
	80 (60–120)	80 (60–95)	
Creatinine clearance (ml/min)	68 ± 48	97 ± 57	<0.005
	53 (4–208)	86 (14–219)	
(G/l)	6.7 ± 2.0	8.4 ± 2.3	<0.001
	6.6 (3.2–13.0)	8.5 (5.0–13.4)	
Proteinuria (g/day)	1.4 ± 2.0	1.6 ± 1.8	n.s.
	0.7 (0–10.5)	1.0 (0–7.0)	
Haematuria—none	44%	48%	n.s.
+	25%	16%	
++	16%	14%	
+++	15%	22%	
Patients receiving immunosuppression[a]	8%	24%	<0.05
	(2 of 100)	(9 of 37)	

DBP, diastolic blood pressure; n.s. = not significant.
Mann–Whitney U-test P values are shown except for haematuria, which was assessed by the χ^2 test. The data are given as means ± SD in the upper rows and medians (minimum–maximum) in the lower rows.
[a]All patients receiving immunosuppression had corticosteroids as mono- or combination therapy.

of high PDGF-DD concentrations with higher creatinine clearance and high leukocyte counts (Table 4). In addition, high PDGF-DD levels were associated with lower diastolic blood pressure and the administration of immunosuppression (Table 4). In contrast, no significant differences evolved for potential markers of disease activity, i.e. the extent of proteinuria or haematuria, in the two groups (Table 4).

Attempts to detect PDGF-DD in urine samples of these patients consistently failed (data not shown).

Individual course of PDGF-DD levels during the follow-up in patients with IgAN

Multiple samples (median 4, range 1–16 samples per patient) were available in the majority of patients with IgAN during 16 ± 18 months of follow-up. As shown in Figure 4, in those patients for whom four or more samples were available, most patients exhibited relatively little fluctuation over time and individual coefficients of variation were $21 \pm 7\%$ (range 8–32%). Of note, the patient with the highest PDGF-DD level in Figure 4, i.e. 3.68 ng/ml, had no evidence of renal disease activity at the time of the serum sample (i.e. normal creatinine clearance, proteinuria below 200 mg/day, no haematuria, normal CRP) or at the time of the next sample, which was obtained 6 months later. During the follow-up, the clinical features of these IgAN patients remained stable in the majority of cases.

PDGF-DD serum levels in other disease entities

We failed to detect any association between PDGF-DD serum levels and any of the measured parameters in patients with MN or VAS. In patients with FSGS, PDGF-DD

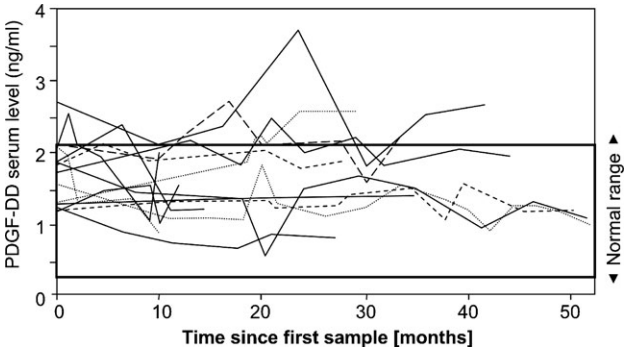

Fig. 4. Individual courses of PDGF-DD serum levels in patients with IgAN during 0–52 months of follow-up. Only those patients ($n = 14$) for whom four or more serum samples were available for measurements entered the graph.

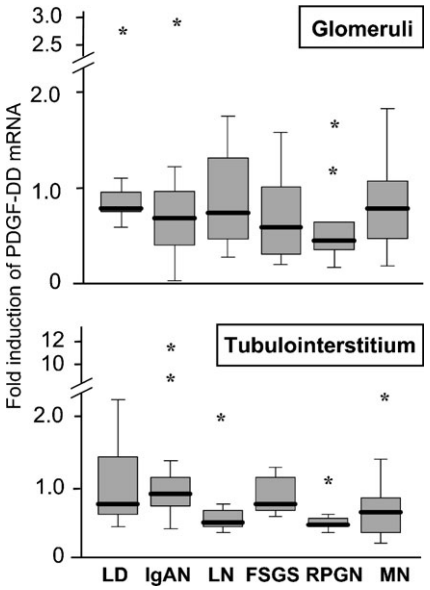

Fig. 5. Box-plots of intrarenal expression of PDGF-DD mRNA in patients with glomerular diseases. We found no significant differences in the PDGF-DD mRNA expression in microdissected glomeruli or the tubulointerstitium in healthy living kidney donors (LD; $n = 9$), patients with IgA nephropathy (IgAN; $n = 24$), lupus nephritis (LN; $n = 11$), focal segmental glomerulosclerosis (FSGS; $n = 13$), rapidly progressive glomerulonephritis (RPGN; $n = 9$) or with membranous nephropathy (MN; $n = 22$). PDGF-DD mRNA data shown are normalized to the reference gene GAPDH. Levels in healthy kidney donors were set as 1. Outliers are marked with asterisks.

was positively correlated with the urinary protein to creatinine ratio ($R = 0.786$, $P = 0.036$, $n = 7$), urinary albumin to creatinine ratio ($R = 0.762$, $P = 0.028$, $n = 8$) as well as LDH ($R = 0.881$, $P = 0.004$, $n = 8$). In patients with LN, we found PDGF-DD to be negatively correlated with serum total protein ($R = -0.489$, $P = 0.040$, $n = 18$) and positively correlated with 24-h proteinuria ($R = 0.587$, $P = 0.035$, $n = 13$) and haematuria ($R = 0.597$, $P = 0.024$, $n = 14$).

Intrarenal expression of PDGF-DD mRNA

To investigate intrarenal synthesis of PDGF-DD at the mRNA level, microdissected glomeruli and tubulointerstitium were examined. Compared to normal glomeruli obtained from living kidney donors, we found no difference in PDGF-DD mRNA expression in glomeruli of patients with IgAN, LN, FSGS, MN or rapidly progressive GN (Figure 5). We also found no alteration in tubulointerstitial PDGF-DD transcript numbers in the same set of patients (Figure 5).

Discussion

To our knowledge, only two studies so far have reported on PDGF-DD levels in humans. In the first study, 50 normal controls mostly exhibited mean serum values of <4 ng/ml, which was the sensitivity limit of the ELISA [6]. Only three patients were reported to have PDGF-DD serum levels >10 ng/ml [6]. Whereas such high values were not observed in our normal population using a different ELISA, the normal range determined in the present study, i.e. 0.25–2.09 ng/ml, is most compatible with these prior observations. In that first study, patients with ovarian, renal, lung, breast and brain cancer frequently exhibited serum PDGF-DD levels >10 ng/ml [6], which was also confirmed in the present study in patients with lung cancer and thus served as a positive control. A second study, published in Chinese, also described increased serum and urinary PDGF-DD levels in children with IgAN when compared to healthy controls [8]. In contrast to the present study, PDGF-DD was positively correlated with proteinuria and was upregulated in renal biopsies of IgAN children.

The major observation of the present study was that patients with IgAN exhibited raised serum concentrations of PDGF-DD in ~30% of the samples obtained. The increases were generally mild and not comparable to the situation in an experimental model of mesangioproliferative GN (anti-Thy 1.1 nephritis), where at the peak of disease activity we found PDGF-DD protein levels of 27.7 ± 14.5 ng /ml as compared to the levels in normal rats. The latter were consistently below the detection limit (<0.02 ng/ml) [2]. Apart from species and assay differences, a likely explanation to reconcile these data is that the anti-Thy 1.1 model is a fulminant and synchronized model of mesangioproliferative GN, whereas the course of human mesangioproliferative IgAN is much more variable and insidious. Our data emphasize the caveats of extrapolating from experimental data to clinical practice and urge for the development of animal models that would more closely mimic the slow course of human IgAN. Also, in the human disease, kinetics of disease activity, e.g. chronic low-grade versus repetitive bouts, are largely unknown since no reliable non-invasive laboratory markers exist for its assessment. Importantly, both proteinuria and microhaematuria may indicate both disease activity and glomerular defects following phases of activity. The latter may explain why we failed to detect significant differences in the extent of proteinuria and haematuria when we compared samples with normal and elevated PDGF-DD levels. The weak negative correlation between PDGF-DD levels and proteinuria, which only became apparent when all patients with glomerular diseases were pooled,

most likely indicates urinary losses of PDGF-DD in heavy proteinuria.

A second insight gained by our study is the positive correlation of PDGF-DD serum levels with creatinine clearance in both patients with IgAN and the whole patient population. In the whole population, this correlation largely depended on the IgAN patients, since the statistical significance of the correlation was lost when only the sera of patients with non-IgAN diseases were assessed (data not shown). The particularly notable feature, therefore, is that unlike most other low-molecular-weight proteins, PDGF-DD (molecular weights 22–47 kDa) did not appear to accumulate in serum with advancing renal failure. In contrast to PDGF-DD, serum levels of PDGF-AB (molecular weight 28–35 kDa) increased ∼20–100% in advanced renal failure [9,10]. Also, in contrast to PDGF-BB and -AB [11], no association between increased PDGF-DD levels and hypertension evolved in the present study.

A third observation of our study was the positive correlation of PDGF-DD serum levels with leukocyte counts in both whole renal disease population and in patients with IgAN. While we are not aware of studies specifically investigating circulating leukocyte content of PDGF, it was reported that PDGF-DD is expressed by synovial macrophages of patients with rheumatoid arthritis and in macrophages of atherosclerotic plaques [12,13]. In addition, PDGF-DD mRNA has been detected in peripheral blood leukocytes [14]. Given the correlation of PDGF-DD levels with leukocyte counts, these may play a role as origin of PDGF-DD synthesis. Release of PDGF-DD by circulating leukocytes could also contribute to our observation that patients with corticosteroid-induced leukocytosis exhibited elevated PDGF-DD serum levels (Table 4).

Fourthly, we noted a dissociation of PDGF-DD serum levels and intraglomerular PDGF-DD synthesis, in that serum levels were elevated in IgAN patients and decreased in LN patients, whereas intrarenal PDGF-DD mRNA content did not differ from that of healthy kidneys in either disease entity. In prior systematic studies, PDGF-DD mRNA expression was high in adrenal glands, pancreas, adipose tissue, mammary glands, testis and stomach [5]. Somewhat lower expression was found in the heart, trachea, bladder and adult and fetal kidneys [5]. The only data on PDGF-DD protein expression in human renal disease was provided by Taneda *et al*., who investigated patients with obstructive nephropathy and found PDGF-DD overexpression in the tubulointerstitum, co-localized with α-smooth muscle actin-positive cells [15]. In another study, the same group noted in normal adult human kidney the expression of PDGF-DD protein in podocytes, vascular smooth muscle cells and some neointimal cells of arteriosclerotic intrarenal vessels [16]. Recently, PDGF-DD expression was found in glomeruli but not in tubulointerstitium in kidney biopsies from healthy subjects and patients with chronic allograft nephropathy or acute vascular rejection. PDGF-DD expression was similar in all three groups examined [17]. However, at least in our hands, the precise intrarenal localization of PDGF-DD up-regulation has remained elusive, since extensive attempts to immunostain PDGF-DD in human kidney sections were not successful. Posttranscriptional regulation of PDGF synthesis has not been described so far, and thus it is unlikely that our RNA-based analysis might have missed a major regulation of the intrarenal PDGF-DD mRNA synthesis. On the other hand, renal posttranslational regulation of PDGF-DD activity cannot be ruled out since PDGF-DD is secreted as an inactive homodimer which needs proteolytical cleavage to become activated [5]. Our data thus suggest that the source of the elevated PDGF-DD levels in patients with IgAN resides outside the kidneys. This might have implications for the pathogenesis of IgAN since the extrarenal overexpression of PDGF-DD in mice induced a pronounced proliferation of mesangial cells [3].

Another important observation of this study is that the majority of patients (10 of 18) with lupus nephritis had decreased serum PDGF-DD levels. Of note, the only two lupus patients with elevated serum PDGF-DD levels exhibited class VI lupus nephritis. Glomerular and tubulointerstitial mRNA expression did not differ from that of healthy living donors, arguing for an extrarenal source of systemic PDGF-DD down-regulation. Decreased PDGF-DD serum levels in LN patients did not relate to leukopenia, as all enrolled patients exhibited numbers within or above the normal range (data not shown). Possibly low levels of PDGF-DD in LN patients were related to immunosuppressive therapy, since all LN patients received intensive immunosuppressive treatment, whereas this was the case in only 18% of IgAN patients and 30–42% of patients with other GNs. The relevance of low serum PDGF levels in lupus remains speculative. PDGF, including PDGF-DD, acts as a potent chemoattractant for monocytes and macrophages [13]. *In vitro*, T cells stimulated with PDGF-BB produced increased interleukin (IL)-2 and reduced IL-4, IL-5 and interferon (IFN)-γ, suggesting that PDGF is a potent regulator of T-cell function [18]. In line with this, imatinib mesylate, an inhibitor of c-abl, PDGFR and c-kit protein tyrosin kinases, was shown to inhibit dendritic cell differentiation and their ability to activate proper T-cell responses *in vitro* [19]. In a mouse model of lupus nephritis, imatinib treatment significantly reduced mortality and renal injury [20]. Thus, although some indications exist that PDGF may play an important role in the regulation of the immune system, the data are still inconclusive.

In summary, we present the first evidence for elevated concentrations of circulating PDGF-DD levels in a subset of adult patients with IgAN. Our data thereby further support the rationale for anti-PDGF-DD therapy in mesangioproliferative GN. Indeed, it has recently been shown in a clinical phase I study that administration of a neutralizing monoclonal antibody to PDGF-DD can reduce PDGF-DD serum concentrations in normal persons to non-detectable levels for prolonged periods [21]. We also provided the first evidence that serum PDGF-DD levels are decreased in patients with LN. The role of such decrease remains elusive and may be elucidated further in studies assessing PDGF-D serum levels before and after induction therapy in LN.

Acknowledgements. Materials for the PDGF-DD ELISA were kindly provided by Curagen Corp., Branford, CT, USA. We thank Dr Loic Giot and Dr William J. LaRochelle for their comments on the manuscript. We are grateful to Dr Ulf Eriksson for providing the polyclonal anti-PDGF-DD antibody for western blotting. The excellent technical assistance of Gabriele Dietzel and Anneliese Michaelis is gratefully acknowledged. This

work was supported in part by grants SFB542, project C4 and C7 from the Deutsche Forschungsgemeinschaft (DFG) to PM, TO and JF, respectively, and the Else Kröner-Fresenius Foundation to CDC. We thank all participating centres of the European Renal cDNA Bank-Kroener-Fresenius biopsy bank (ERCB-KFB) and their patients for their cooperation. Active members at the time of the study: C. D. Cohen, M. Fischereder, H. Schmid, L. Weber, M. Kretzler, D. Schlöndorff, Munich; J. D. Sraer, P. Ronco, Paris; M. P. Rastaldi, G. D'Amico, Milano; P. Doran, H. Brady, Dublin; D. Mönks, C. Wanner, Würzburg; A. J. Rees, Aberdeen; F. Strutz, G. A. Müller, Göttingen; P. Mertens, J. Floege, Aachen; N. Braun, T. Risler, Tübingen; L. Gesualdo, F. P. Schena, Bari; J. Gerth, G. Wolf, Jena; R. Oberbauer, D. Kerjaschki, Vienna; B. Banas, B. K. Krämer, Regensburg; M. Saleem, Bristol; H.-P. Marti, R. P. Wüthrich, Zürich; W. Samtleben, Munich; H. Peters, H. H. Neumayer, Berlin; M. Daha, Leiden; K Ivens, B. Grabensee, Düsseldorf; F. Mampaso(†), Madrid; J. Oh, F. Schaefer, M. Zeier, H.-J. Gröne, Heidelberg; P. Gross, Dresden; G. Tonolo; Sassari; V. Tesar, Prague and H. Rupprecht, Bayreuth.

Conflict of interest statement. J.F. consultancy for Curagen Corp., otherwise nothing to declare.

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Received for publication: 21.11.08; Accepted in revised form: 13.3.09